Concomitant LPS induced transfer of blood derived components including imunoglobulins into milk

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INTERPRETATIVE SUMMARY

Concomitant LPS induced transfer of blood derived components including immunoglobulins into milk. By Lehmann et al.

By the use of animals with manipulated blood BHBA concentration this study confirms the expected blood origin of a number of factors that increase in milk during the early phase of LPS-induced mastitis and describes the time course of their increase relative to that of somatic cell count. The milk concentration of L-lactate, BHBA, lactate dehydrogenase, IgG_1, and IgG_2 increased concomitantly in the milk of LPS-challenged quarters suggesting their blood origin. Many of these blood components leak into milk but are not necessarily involved in mammary immune defence.

BLOOD COMPONENTS IN MILK DURING MASTITIS

15 **Concomitant LPS induced transfer of blood derived components including**

16 **immunoglobulins into milk**

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ABSTRACT

During a mammary immune response the integrity of the blood milk barrier is negatively affected and becomes leaky. The aim of the present study was to demonstrate the blood origin, and to investigate changes in the concentration, of various constituents including immunoglobulins in blood and milk during the early phase of a lipopolysaccharide (LPS) induced mastitis. Five lactating dairy cows received continuous β-hydroxybutyric acid (BHBA) clamp infusions to maintain elevated BHBA blood concentrations (1.5 - 2.0 mmol/L) from 48 h before and 8h after LPS administration. One udder quarter was infused with 200 µg of *E. coli* LPS. A second quarter served as control. Milk and blood samples were taken hourly for 8h post-challenge (PC). The somatic cell count (SCC) in LPS challenged quarters was increased from 4 h PC until the end of the experiment compared to control quarters. In LPS challenged quarters, L-lactate, BHBA, LDH, Immunoglobulin (Ig) G_1, and IgG_2 were increased at 3 h PC and remained elevated until the end of experiment (8 h PC) as compared to control quarters. In addition, the optical density (OD) values in milk in a non-quantitative ELISA for antibodies directed against bluetongue virus (used as a measure of non-specific antibody transfer; all animals were vaccinated) increased and thus indicates an increase of these antibodies in response to LPS treatment. L-lactate concentration also increased in blood 2 h PC and in the milk of control quarters during the experiment from 3h PC. A second experiment was conducted in vitro to investigate a possible contribution from destructed milk cells on L-lactate and activity of LDH in milk. Aliquots of milk samples (n=8) were frozen (-20 °C) or disrupted with ultrasound, respectively. Freeze thawing and ultrasound treatment increased LDH in milk samples, but had no effect on L-lactate concentrations. Results suggest that intramammary infusion of LPS induces a systemic response as evidenced by an elevation of blood L-lactate. The concomitant changes of all investigated components suggest that they were blood-derived. However, the increase of

blood components in the milk is not necessarily supportive of the mammary immune system, and likely a side effect of reduced blood-milk barrier integrity.

Key words: blood milk barrier, IgG, lactate, BHBA

INTRODUCTION

In the bovine mammary gland, invading pathogens induce an immune response by stimulating the innate immune system. It is generally accepted that the main effectors of the mammary immune defense against mastitis-causing bacteria are blood derived polymorphonuclear neutrophils (**PMN**) (Burton and Erskine, 2003). Their infiltration into the mammary gland and their accumulation in milk is measured as an increase of the milk somatic cell count (**SCC**), which is widely used as a measure of hygienic milk quality (Harmon, 1994). During the early acute stage of mammary inflammation a leakage of blood constituents occurs because of an alteration in the blood milk barrier (Burton and Erskine, 2003). In addition to SCC, the concentrations of a number of other components change in milk in response to inflammation of the mammary gland, not all of which contribute to host-defence.

Milk L-lactate (hereafter referred to as **lactate**) and the enzyme, lactate dehydrogenase (**LDH**) increase in milk during intramammary infection and have been considered indicators of mastitis even though the immunological contribution of these factors is not known (Davis et al., 2004; Chagunda et al., 2006). Immunoglobulin (**Ig**) G is the major immunoglobulin in ruminant milk (Butler, 1983) and consists of two subclasses, IgG_1 & IgG_2. IgG_1 is the predominant antibody type in milk from healthy quarters because of an active, selective IgG_1 transport across the blood milk barrier likely mediated by the FcRn receptor system (Baker et al., 2009). In mastitic milk IgG_2 increases in concentration (Caffin and Poutrel, 1988) and plays a critical role in mammary immune defense against mastitis pathogens (Burton and Erskine, 2003). However, antibodies which are not directed against mastitis-causing pathogens are also nonspecifically transferred into the mammary gland and can be found in the milk of healthy udders, e.g. antibodies against the bovine viral diarrhea (BVD) virus or those against bluetongue virus (Kramps et al., 1999; Kramps et al., 2008). After the bluetongue disease outbreak in Northern Europe in 2006, Swiss cows were vaccinated against the bluetongue virus serotype 8 (BTV-8) between 2008 and 2010.

Another blood constituent without any obvious benefit for immune defense in the mammary gland is the ketone body, β-hydroxybutyrate (**BHBA**), which is produced by the liver. A significant correlation of 0.66 between BHBA in blood and milk was described by Enjalbert et al. (2001), however information on its possible role in mammary gland health was not provided. Intramammary administration of lipopolysaccharide (LPS) from *Escherichia coli,* a common mastitis pathogen, is a well established method to experimentally induce mastitis under defined conditions for studying the immune response of the mammary gland (Schmitz et al., 2004; Baumert et al., 2009; Wellnitz et al., 2011). In these studies LPS from different *E. coli* strains were shown to require different concentrations in order to induce comparable immune responses of the mammary gland. This effect is, besides differences in individual responsiveness, due to variations in the fine structure of LPS (Rietschel et al., 1994).

The objective of the present study was to document changes in milk SCC and lactate, BHBA, LDH, IgG_1, and IgG_2 in milk and to contrast these changes to those in blood during the first hours of LPS induced mastitis. We hypothesized that the increase of lactate, along with that of other components that are expected to originate from blood, is a result of loss of the integrity of the blood milk barrier. An additional experiment was performed to establish the origin of lactate in milk.

MATERIALS AND METHODS

Experiment 1: LPS induced mastitis

Animals and management

Five mid-lactating dairy cows (Holstein) in their 3rd to 4th lactation were used in these studies. While maintained in a tie stall, foremilk samples were taken from each quarter approximately three weeks before, one week before and immediately before the start of the experiment to verify a constantly low SCC (<150,000 cells/mL). A DeLaval cell counter, (DCC) (DeLaval,

Tumba, Sweden), was used to measure SCC. All cows had been immunized against the bluetongue virus serotype 8 (Bovilis® BTV8, Intervet) approximately one year before the experiment as a part of a national vaccination campaign.

Experimental design

DL-Beta-hydroxybutyric acid solution (2.14M; Sigma-Aldrich, H6501) was infused 48 h before and during the LPS challenge to induce a hyperketonemia (1.5 to 2.0 mmol/L of BHBA in the plasma) throughout the experiment as described by Zarrin et al. (2011). Two intravenous catheters (Cavafix® Certo® Splittocan®, B. Braun Melsungen AG, Germany) inserted one in each jugular vein were used to infuse BHBA and to take blood samples, respectively.

On the day of challenge and after the morning milking, cows were intra-mammarily infused with 200 µg *E. coli* LPS into the left front quarter (LPS from *E .coli* serotype O26:B6, Sigma Aldrich, Buchs, Switzerland) diluted in 10 mL of sterile saline solution (9 g/L). The right front quarter was used as a control and treated with 10 mL of the saline solution. Milk samples (~20mls) were hand collected for an immediate SCC measurement that continued hourly for 8 hours post-challenge from the experimental quarters after discarding the first three squirts of milk. Milk samples were frozen at -20 °C for later analyses. Skim milk was prepared by centrifugation at 4,000 x *g* for 15 min and milk serum was prepared by centrifugation of the skim milk at 14,000 x *g* for 30 min.

Blood samples were collected at times coinciding with milk sample collection. Blood was collected into tubes containing FE Sodium Fluoride/tri-potassium-EDTA (Greiner Bio-One, Kremsmünster, Austria) as anticoagulant. Tubes were stored briefly on wet ice and then centrifuged for 20 minutes at 3,000 x g. Plasma was collected and frozen at -20 °C until analyses.

144 LDH activity, lactate concentration, and BHBA concentration were measured in milk serum
145 and blood plasma using the test kits LDH IFCC (Axon Lab AG, Baden, Switzerland), Lactate
146 PAP (bioMérieux, Marcy l`Etoile, France) and Ranbut (Randox Laboratories, UK),
147 respectively, with an automated analyzer (COBAS MIRA, Roche Diagnostics, Switzerland)
148 according to the manufacturer's instructions.
149 The IgG_1 and IgG_2 concentrations in skim milk and blood plasma were measured using
150 bovine IgG_1 and IgG_2 ELISA quantitation kits (Bethyl Laboratories, Montgomery, USA)
151 according to the manufacturer`s instructions with the following slight modifications.
152 After coating of plates overnight with anti-bovine IgG_1/ IgG_2 the plates were washed using an
153 automatic washing system (microplate washer Columbus Pro, Tecan, Männedorf,
154 Switzerland) and subsequently blocked with 150 µl/well of 5 % fish skin gelatin (Sigma
155 Aldrich, Buchs, Switzerland) in distilled water for 60 min at room temperature. After
156 washing, 100 µl of skim milk diluted in washing buffer (1:100 for IgG_2 and 1:1000 for IgG_1)
157 or blood plasma samples were added to each well. On the plate, the samples were diluted in
158 three serial 1:2 dilutions using a multichannel pipette. One control sample was added to each
159 plate and was used for the calculation of coefficients of variations that reached 10 and 20%
160 within, and between assays, respectively. Plates were incubated for 60 min at room
161 temperature and subsequently washed as described above. Sheep anti-bovine IgG_1/ IgG_2 HRP
162 conjugated antibody were diluted 1:15,000 and 1:10,000, respectively in wash buffer and 100
163 µl of this solution was added to each well. The concentrations of IgG_1 and IgG_2 were
164 calculated by extrapolating from a standard curve generated by a Thermomax microplate
165 reader (Molecular Devices, Basel, Switzerland).
166 The ELISA test kit INGEZIM BTV-MILK 12.BTV.K1 (Inmunologia y Genetica Aplicada,
167 S.A., Madrid, Spain) was used to determine bluetongue antibodies in skim milk according to
168 manufacturer`s instructions. OD values were measured at 450 nm by using the Thermovax
169 microplate reader (Molecular Devices, Basel, Switzerland).

170

171 *Data analysis*

172 Data are presented as means ± SEM. Data for SCC, lactate, and LDH activity were log
173 transformed (log10) to improve normality. Differences between LPS and control within each
174 time point and differences between time points within one group were tested for significance
175 ($P < 0.05$) by analysis of variance using a MIXED procedure of SAS (SAS Institute Inc.,
176 Cary, NC, USA, 2002-2008, Release 9.2). The model included time, treatment (LPS or
177 control), the quarter within cow, and their interaction as fixed effects and the cow as repeated
178 subject. For antibodies against bluetongue in milk OD values are presented in a descriptive
179 way.

180

181 ***Experiment 2: milk cell destruction***

182 *Experimental design*

183 To investigate the effect of milk cell destruction on the lactate concentration and LDH activity
184 in milk, eight quarter foremilk samples from 2 different cows were collected during one
185 morning milking. Three aliquots of each milk sample were treated differently; one aliquot was
186 frozen at -20 °C, a second was treated with ultrasound for 10 s at 100 watt (Branson Sonifier
187 150), and the third served as a control without any treatment (stored in the refrigerator at 4°C).
188 Catalytic activity of LDH and lactate concentration were then detected in milk serum,
189 prepared as described previously, using the test kits LDH IFCC (Axon Lab AG, Baden,
190 Switzerland) and Lactate PAP (bioMérieux, Marcy l`Etoile, France), respectively, in an
191 autoanalyzer (COBAS MIRA, Roche Diagnostics, Switzerland) according to the
192 manufacturer's instructions.

193

194 *Data analysis*

Data are presented as means ± SEM. For evaluation of changes of lactate and LDH through freezing and ultrasound, differences between treated and untreated samples were used. Differences between the treatments were tested for significance ($P < 0.05$) by analysis of variance using a MIXED procedure of SAS. The model included treatment as a fix effect and the sample as repeated subject.

RESULTS

Experiment 1: LPS induced mastitis

Somatic Cell Count SCC started to increase in LPS treated quarters at 3 h post challenge (**PC**) and was significantly and continuously elevated from 4h PC until the end of the experiment compared to control quarters (Figure 1A). Control quarter SCC was significantly elevated only at 2 h PC when compared with 0 h.

Lactate The lactate concentration in milk of LPS treated and control quarters (Figure 1B) was increased at 3 h PC ($P < 0.0001$ and $P < 0.05$, resp.) and reached concentrations similar to those observed in blood plasma (>1mM) by 6h PC, reaching a maximum at 7h PC. This increase in lactate was greater in LPS treated quarters as compared to control quarters ($P<0.05$). In two cows the lactate concentrations in milk of LPS treated quarters exceeded the values of those in blood. In the blood plasma, lactate concentration was higher at 2 h PC as compared to the concentration at 0 h ($P < 0.05$) and continued to increase steadily until the end of the experiment at 8 h PC.

BHBA In 25 out of 90 milk samples BHBA concentrations were below the detection (Figure 1C). In such cases half of the detection limit (0.05 mmol/L) was then used as a value for mathematical and statistical calculations. Significantly higher BHBA concentrations were identified in LPS quarters compared to control quarters from 3 h PC and continued to increase until the end of the experiment. Control quarter BHBA concentration did not change. The

221 BHBA blood concentration was not analyzed because it was controlled via a BHBA clamp
222 infusion. At 0 h the BHBA concentration in blood plasma was 16 fold higher ($P < 0.05$) than
223 in the milk. During the experiment this ratio diminished to a 3 fold difference at 8 h PC, but
224 did not reach the blood plasma concentrations during the experiment.
225 **LDH** The LDH activity in milk of LPS treated quarters (Figure 1D) was significantly higher
226 at 3 h PC compared to control quarters and remained elevated until the end of the experiment,
227 but did not reach blood concentrations. The activity of LDH in blood plasma and milk from
228 control quarters did not change throughout the experiment. At t = 0 h the blood/milk LDH
229 ratio was 29:1 decreasing during the experiment to be 3:1 at 8 h PC.
230 **IgG_1 and IgG_2** From 3 until 8 h PC IgG_1 and IgG_2 concentrations in milk of LPS treated
231 quarters were significantly higher compared to control quarters (Figure 1E, Figure 1F). No
232 changes in IgG1 and IgG2 concentrations in blood plasma and milk of control quarters could
233 be detected throughout the experiment. The blood:milk IgG1 and IgG_2 ratios were 77:1 and
234 554:1, respectively, before LPS challenge. At 8 h PC the blood:milk IgG_1 and IgG_2 ratios
235 diminished to 17:1 and 21:1, respectively. In addition, the ratio of IgG1:IgG2 in blood plasma
236 and milk of treated and control quarters was calculated throughout the experiment (Figure 2).
237 At t=0 h the ratio in milk from both treated and control quarters was approximately 4 while in
238 blood the ratio was 0.48. In the blood plasma the ratio IgG_1:IgG_2 was 1.0:2.1 and did not
239 change throughout the experiment. In the milk of the LPS treated quarter, the ratio started to
240 decrease at 2 h, and reached the level of blood plasma at 3 h PC which was maintained until
241 the end of experiment.
242 **Anti-bluetongue virus antibodies** The non-quantitative test indicated that all examined milk
243 samples were positive for anti-bluetongue virus antibodies (cut-off OD value for a positive
244 result was 0.38). The OD values between 0 and 2 h PC ranged from 0.89 to 1.46 in control
245 quarters and from 0.87 to 1.49 in LPS challenged quarters. Between 3 and 8 h PC the OD

values remained ranged still from 0.92 to 1.46 in control quarters but from 1.17 to 1.74 in LPS challenged quarters.

Experiment 2: milk cell destruction

Freezing and thawing and ultrasound treatment significantly increased the catalytic activity of LDH in milk by 44 and 50%, respectively. Freezing and thawing and ultrasound treatment had no effect on lactate concentrations in milk. (Figure 3)

DISCUSSION

In the present study changes in various blood and milk components were quantified during the early phase of a LPS induced mastitis. The observed increase in SCC in response to intramammary LPS challenge has been shown before (Baumert et al., 2009; Wellnitz et al., 2011). The slight increase of SCC in the 2 first hours in all quarters was also expected and regularly observed after milking (Wellnitz et al., 2011). Coinciding with the infiltration of PMN, the blood milk barrier is expected to become leaky allowing blood constituents to enter the lumen (Burton & Erskine, 2003) or, as has been shown for lactose, exit the lumen and enter the circulation (Bruckmaier et al., 2004).

The hypothesis that increased passage of blood constituents into milk occurs during mammary inflammation was supported by the present study. The increasing BHBA in milk after LPS challenge must have been blood derived because it occurred only in animals which received a BHBA infusion to keep blood BHBA concentrations constantly elevated throughout the experiment. In cows with normal BHBA blood concentrations the concentration in milk remained below the detection limit for both LPS-treated and control quarters (0.1 mmol/L; data not shown). The accumulation of BHBA in milk of LPS-treated quarters suggests that LPS-induced mastitis causes a loss of the blood milk barrier integrity (Bannerman et al.,

2004) and allows the BHBA molecules to pass through the barrier and to accumulate in milk. These results are in agreement with Nielsen et al. (2005) who found higher BHBA milk concentrations in diseased quarters compared to healthy quarters. The other investigated parameters lactate, LDH, and immunoglobulins started to increase at the same time after LPS challenge as BHBA which indicated that also these factors do at least in part originate from blood.

Experiment 2 showed that milk cell disruption (by freezing or by ultrasound) leads to increased LDH activity in milk in agreement with earlier studies (Lipperheide et al., 1995; Kato et al., 1989) whereas lactate did not significantly change. Milk LDH activity was thought to originate from the blood and to be a suitable indicator of increased permeability of the blood milk barrier (Symons and Wright, 1974). In experiment 1 blood:milk LDH ratio decreased in response to LPS challenge in a similar manner to that observed for BHBA, yet milk concentrations did not reach those observed in blood within the experimental period. This is possibly due to the large difference in the molecular weights (140 KDa vs 104). Our current finding suggests that the origin of LDH in milk may be attributable to both soluble LDH from blood as well as disrupted leukocytes (Kato et al., 1989) and epithelial cells (Bogin et al., 1977; Zank and Schlatter, 1998).

Lactate is known to increase in milk during mastitis (Davis et al., 2004). However, the origin of the lactate remained unclear. Our data demonstrated that lactate concentrations increase also in blood in response to intramammary LPS administration. Intravenous LPS administration has been shown to increase blood lactate concentrations in cows (Giri et al., 1990). In the present study lactate concentration in milk of control quarters increased slightly but concomitantly with blood lactate concentration. This is in contrast to SCC, BHBA, LDH, IgG_1, IgG_2, and anti-bluetongue virus antibodies which remained relatively stable in blood. This underlines the suggestion that the majority of lactate in healthy quarters originates from blood.

298 Lactate concentrations in milk of LPS-treated quarters increased to comparable levels to those
299 in blood plasma (at 6 h PC), and, surprisingly, in two cows the concentrations of lactate in
300 milk of LPS treated quarters exceeded the concentrations of lactate in blood. This is in
301 contrast to the concentration of BHBA and LDH which was always higher in blood than in
302 milk. This observation may be explained by additional lactate production and release during
303 anaerobic metabolism by milk and epithelial cells in the gland (Silanikove et al., 2011; Mayer
304 et al., 1988) whereas the results of experiment 2 showed that the lactate produced by cells in
305 the milk appears to be released from these cells directly after synthesis. In addition, the small
306 size of the lactate molecule (90Da) as compared to the other investigated constituents may
307 allow for increased transfer from blood into milk. The existence of a specific transmembrane
308 transport mechanism for lactate via aquaporins is likely since aquaporins transport lactate
309 (Conde et al., 2010), and aquaporins are present in the bovine mammary gland (Mobasheri et
310 al., 2011).
311 In the present study IgG_1 was the predominant IgG antibody subclass in milk before LPS
312 challenge which is in agreement with earlier studies (Guidry et al., 1980; Butler, 1983). It is
313 likely that the specific transport mechanism for IgG_1 which is prevalent prepartum colostrum
314 formation, remains weakly expressed during lactation. Both IgG_1 and IgG_2 increased
315 simultaneously three hours after intramammary LPS challenge and the IgG_1:IgG_2 ratio
316 reached a similar milk ratio to that observed in blood. IgG_2 is considered to be the main
317 opsonic antibody for neutrophil phagocytosis and is, therefore, most important for mammary
318 immune defense against mastitis pathogens (Hill et al., 1983; Leitner et al., 2000). Both IgG_1
319 and IgG_2 milk concentrations increased 3 h PC, along with SCC, BHBA, LDH, and lactate,
320 indicating an unspecific transfer through the opening of the blood milk barrier.
321 The results of the used ELISA for the detection of antibodies against blue tongue virus in milk
322 can be interpreted solely as positive, doubtful, or negative according to the manufacturer.
323 However, the higher OD values measured in milk of LPS challenged quarters after 3 h PC

indicate an increase of antibodies against bluetongue virus. Thus, a non-specific accumulation of various antibodies in the mammary gland during mastitis is likely. Obviously, during the alteration of the blood milk barrier, antibodies regardless of specificity, are able to pass the blood milk barrier. This is in agreement with Charlier et al. (2006) who found higher *Ostertagia ostertagi* antibody levels in milk of experimentally infected quarters. They described that an acute mastitis causes a flow of specific and non-specific antibodies from blood into milk. These finding could be important to understand the effectiveness of vaccinations against mastitis pathogens.

CONCLUSIONS

In conclusion, all investigated parameters appear to be blood derived and their increase in milk occurs in parallel with the increase of SCC. The integrity of the blood milk barrier is reduced during the early phase of LPS-induced mastitis. This allows various blood constituents to pass through the blood milk barrier to milk which may or may not support the mammary immune response. Intramammary LPS infusion also induces a systemic immune reaction shown by our evidence of an increase of blood lactate concentration that affects the lactate in milk of control quarters. The increase of some specific IgG in milk during mammary immune response may not be supportive for the mammary immune system, and is at least partially just a result of reduced integrity of the blood-milk barrier. However, these results show that the degree to which the blood milk barrier integrity is compromised by a mastitis pathogen influence milk composition and content of blood components. The combat against mastitis pathogens may be improved by the alteration of the blood-milk barrier especially if specific antibodies are present in the blood.

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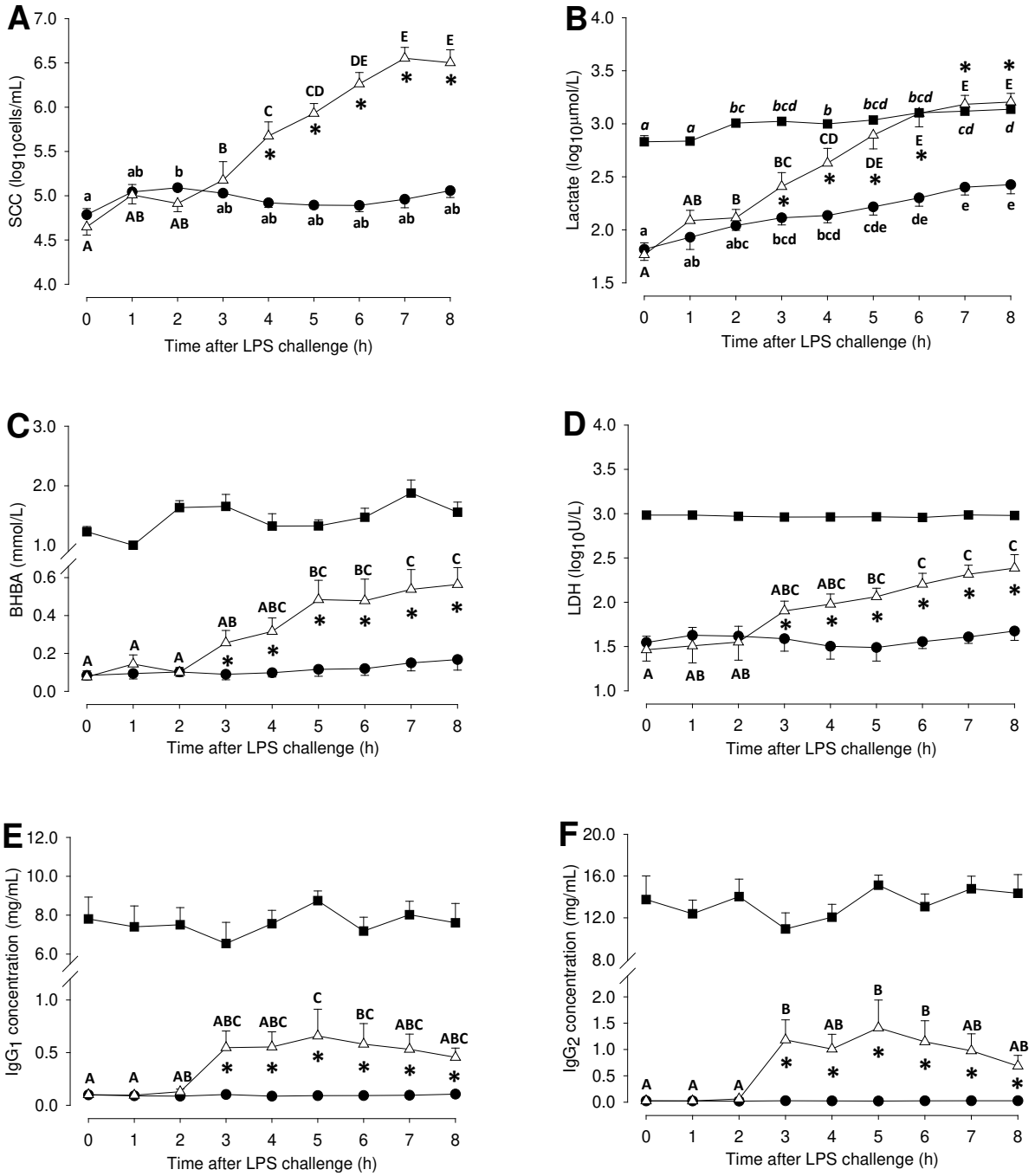
456

Legend of the figures

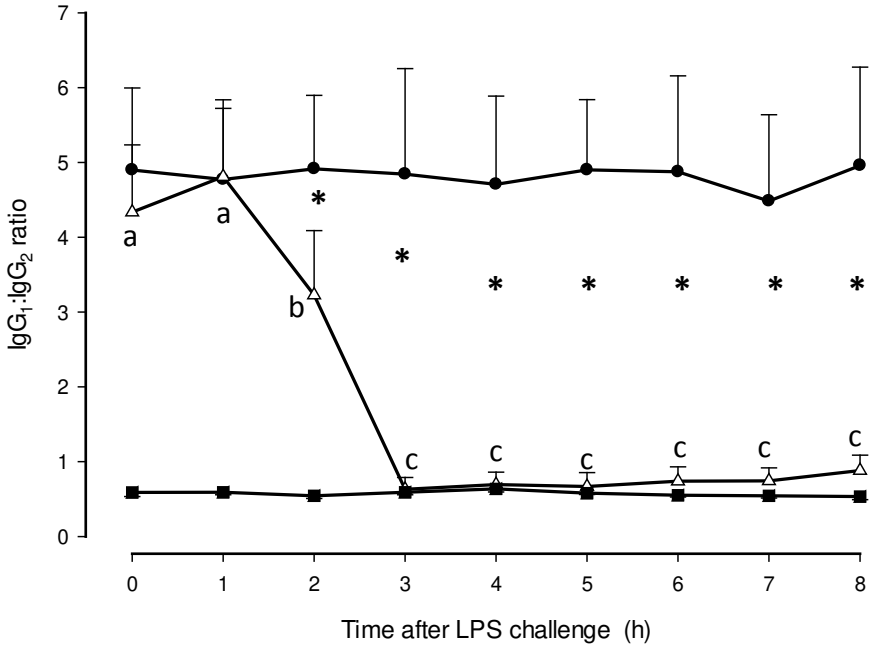
Figure 1 Milk somatic cell count (SCC, A), lactate (B), β-hydroxybutyrate (BHBA, C), lactate dehydrogenase (LDH, D), immunoglobulin G_1 (IgG_1, E), and immunoglobulin G_2 (IgG_2, F) concentrations, in milk of control quarters (●), LPS challenged quarters (△) and in the blood plasma (■) of 5 cows. * indicates a significant ($P < 0.05$) difference between LPS and control quarters within each time point. a,b,c,d,e : different letters indicate significant differences) between time points within control quarters; A,B,C,D,E within LPS challenged quarters; no letters indicate no differences between time points

Figure 2 IgG_1: IgG_2 ratios in blood plasma (■) and milk of control quarters (●) and LPS challenged (△) quarters. * indicates a significant ($P < 0.05$) difference between LPS and control quarters within each time point. a,b,c: different letters indicate significant differences between time points within LPS treated quarters; no letters indicate no differences between time points (control quarters and blood plasma)

Figure 3 Lactate concentration (A) and LDH activity (B) after different treatments (control, -20 °C, and ultrasound) of quarter foremilk samples (n=8). * indicates a significant difference ($P < 0.05$) between freezing or ultrasound treatment and control.



Lehmann et al. Figure 1



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Lehmann et al. Figure 2

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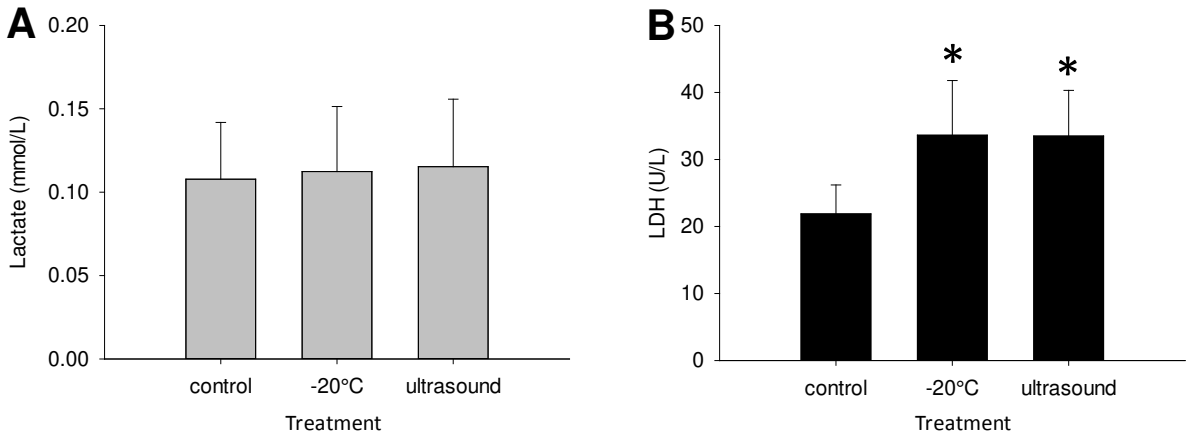

Lehmann et al. Figure 3